



Yin-Yang1 is required for epithelial-to-mesenchymal transition and regulation of *Nodal* signaling during mammalian gastrulation

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ABSTRACT

The ubiquitously expressed Polycomb Group protein Yin-Yang1 (YY1) is believed to regulate gene expression through direct binding to DNA elements found in promoters or enhancers of target loci. Additionally, YY1 contains diverse domains that enable a plethora of protein–protein interactions, including association with the Oct4/Sox2 pluripotency complex and Polycomb Group silencing complexes. To elucidate the *in vivo* role of YY1 during gastrulation, we generated embryos with an epiblast specific deletion of *Yy1*. *Yy1* conditional knockout (cKO) embryos initiate gastrulation, but both primitive streak formation and ingression through the streak is severely impaired. These streak descendants fail to repress E-Cadherin and are unable to undergo an appropriate epithelial to mesenchymal transition (EMT). Intriguingly, overexpression of *Nodal* and concomitant reduction of *Lefty2* are observed in *Yy1* cKO embryos, suggesting that YY1 is normally required for proper *Nodal* regulation during gastrulation. Furthermore, definitive endoderm is specified but fails to properly integrate into the outer layer. Although anterior neuroectoderm is specified, mesoderm production is severely restricted. We show that YY1 directly binds to the *Lefty2* locus in E7.5 embryos and that pharmacological inhibition of *Nodal* signaling partially restores mesoderm production in *Yy1* cKO mutant embryos. Our results reveal critical requirements for YY1 during several important developmental processes, including EMT and regulation of *Nodal* signaling. These results are the first to elucidate the diverse role of YY1 during gastrulation *in vivo*.

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Introduction

Yin-Yang1 (YY1, also called *delta*, NF-E1, and UCRBP) is a multifunctional protein that is thought to alter gene expression through a variety of mechanisms. YY1 contains a GLI-Krüppel zinc finger domain in the C-terminus allowing for direct binding at target loci, which are found throughout the genome often within 1 kb of transcription start sites (Galvin and Shi, 1997; Shi et al., 1991). Functional YY1 binding sequences have also been demonstrated at distant enhancers of many genes including *Snail* and *Otx2* (Palmer et al., 2009; Peinado et al., 2004). YY1 binding sites are highly conserved across the genomes of many species, second only to conservation of SP1, which is itself a binding partner of YY1 in certain cell types (Lee et al., 1993). Despite numerous efforts to functionally define YY1, its endogenous activity remains enigmatic.

YY1 is believed to influence gene activity by inhibiting or strengthening interactions between target loci and other transcriptional activators, such as SP1 and E2F (Dong and Pfister, 1999; Lee et al., 1993; Schlisio et al., 2002; Shi et al., 1997; Yakovleva et al.,

2004). It is also clear that YY1 participates in epigenetic regulation of gene expression through interactions with chromatin modifying complexes such as PRMT1 and members of the Polycomb Repressive Complexes, PRC1 and PRC2 (Garcia et al., 1999; Rezai-Zadeh et al., 2003). Because the known members of PRC2 lack DNA binding capability and the single *Drosophila* ortholog of YY1, *Pho*, has been shown to perform this function, it has been postulated that YY1 is responsible for PRC2 targeting (Brown et al., 1998; Mohd-Sarip et al., 2002). However, it remains unclear if mammalian YY1 does perform this function *in vivo*.

Yy1 has been knocked-out or knocked-down in several species in order to functionally define this dynamic protein. *Pho* homozygous null pupae exhibit impaired pattern formation and aberrant nervous system development (Fritsch et al., 1999; Girton and Jeon, 1994). *Pho* directs PRC2 to target loci, and expression of mammalian *Yy1* partially rescues the *Pho* mutant phenotype (Atchison et al., 2003), indicating that the ability to target PRC2 is at least partially conserved in mammalian YY1. The YY1 paralogues in *Xenopus* are required for gastrulation and neural crest epithelial to mesenchymal transition (EMT), presumably governed at least in part by YY1's role in activation of the *Slug* promoter (Morgan et al., 2004).

In the mouse, preimplantation lethality of *Yy1* null embryos precludes analysis of later embryonic functions (Donohoe et al.,

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1999). Affar et al. demonstrated a dose-dependent necessity for YY1 during mid-gestation, and defined a requirement for YY1 in cytokinesis and cell cycle progression (Affar et al., 2006). Conditional deletion studies have also revealed that YY1 is required for VDJ recombination during pro-B-cell differentiation, as well as oligodendrocyte differentiation and myelination (He and Casaccia-Bonnel, 2008; He et al., 2010; Liu et al., 2007). A role for YY1 has also been documented in male germ cells, where YY1 is critical for double strand break repair and heterochromatin formation (Wu et al., 2009), and deletion of *Yy1* in growing oocytes results in a failure of follicle expansion and paracrine signaling defects in the ovary (Griffith et al., 2011). While these studies have elucidated requirements for mammalian *Yy1* in committed cell types, little is known about its role during gastrulation *in vivo*.

Here we report the developmental and molecular consequences of epiblast-specific deletion of Yin-Yang1, demonstrating a critical role for YY1 in appropriate primitive streak (PS) formation and E-Cadherin repression in cells exiting the streak. Although both mesoderm and endoderm are specified, neither cell type is able to properly migrate. We also observe impaired embryonic to extraembryonic signaling when YY1 is removed from the epiblast, resulting in aberrant morphogenetic movements during gastrulation. Additionally, we show that YY1 is required for negative regulation of *Nodal*, possibly through direct activation of *Lefty2* during gastrulation *in vivo*.

Materials and methods

Embryo production and genotyping

Timing of embryonic development was determined by presence of a vaginal plug the morning after mating (E0.5). Embryos for analysis were generated by mating female mice homozygous for a *Yy1* conditional allele, *Yy1^{lox/lox}* (Affar et al., 2006), to *Yy1^{Δ/WT}*; *Sox2-Cre^{+/+}* males (Hayashi et al., 2002). For visualization of *Yy1* null cells by B-galactosidase staining, females homozygous for both the *Yy1* conditional allele and the R26 reporter (Soriano, 1999) were mated to *Yy1^{Δ/WT}*; *Sox2-Cre^{+/+}* males. At the time of embryo dissection, ectoplacental cone (EPC) DNA extraction was used for PCR genotyping both the *Yy1* (Affar et al., 2006) and *Sox2-Cre* alleles (Hayashi et al., 2002). Embryos were sexed by *Sry* specific PCR using the primers: 5' TTTATGGTGTGGTCCCGTGG3' and 5' CAGCTCTTGCTGTATGTGAT3'. During gastrulation, all genotypes were recovered at expected Mendelian ratios. Mutant embryos are of the genotype *Yy1^{lox/Δ}*; *Sox2-Cre^{+/+}*, hereafter referred to as "mutants" or cKO embryos for simplicity. All other genotypes are referred to as "control" or "wild-type".

RNA extraction and RT-PCR

RNA extraction was performed with Roche High Pure RNA Isolation Kit (Roche 11828665001). cDNA was synthesized with both random hexamers and oligo-dT primers as described previously (Griffith et al., 2011). RT-PCR was performed with 3 μl (1/16 of each embryo) as template for 36 cycles of 30 s at 60 °C, 72 °C and 94 °C with the following gene specific primer pairs (given 5' to 3'): *ActB* (GGCCAGAGCAAGAGAGGTATCC and ACGCAGGATTTCCCTCTCAGC); *Drap1* (GAAATGCCAAAACCATGACC and TTGTCTTTGCCTTTGCTTCC); *Eomes* (CCAGGGTTCTCCGCTCTAC and GTCACCTCCAGCATGTGCAG); *Fgf4* (CTTGCCCTAGTTCTCTGTGCTG and GGAATGATGGGAATGATTGG); *Fgf8* (TGTTGCACTTGCTGTTCTC and ACTCGGACTCTGCTTCCAAA); *Foxa2* (TGAGGTGGGTAGCCAGAAAG and GCTCAGACTCGGACTCAGGT); *Lefty2* (AAGTTTTCAGGGCACTTTTAGGGAC and GGACAA-GCTCACTGAGAATACATCTG); *Nodal* (CGCATCTTCTCTTCAAGC and

GCCTGGTGGAAAATGTCAAT); *Snail* (CTTGCTGTGACGACCTGT and CTCACATCCGAGTGGGTTT); *Tbx6* (GGGACTCAGATCCAGAGCAG and ACTTCAATGCGGATGCTACC); *Twist1* (ACGAGCTGGACTCCAA-GATG and CCTCTGGGAATCTCTGTCCA); *T* (CATGTACTCTTTCTTG-CTGGG and GGTCTCGGGAAGCAGTGGC); *Wnt3* (CGCTCAGCTAT-GAACAAGCA and GGTGTTTCTCCACCACCATC). Quantitative RT-PCR assays were performed using Taqman Gene Expression Assays (*Snail* MM00441533_g1 and *Eomes* MM01351985_m1 multiplexed with *ActB* 4352341E) and Perfecta[®] qPCR SuperMix, Low ROX[™] (Quanta Biosciences # 95052-02K) and run on a Stratagene 3001mx Q-PCR machine using Quanta's recommended cycling conditions.

β-Galactosidase staining

Embryos were fixed for 30 min at room temperature and processed as previously described (Tremblay et al., 2000).

Fixation, embedding, sectioning

Embryos were prepared for histology by fixation in 4% paraformaldehyde (PFA) for 2 h at room temp or overnight at 4 °C. Embryos were dehydrated through a series of methanol washes; 20 min each in 25%, 50%, 75% methanol diluted in phosphate buffered saline/0.01% tween20 (PBT), followed by two 100% methanol washes. Embryos were embedded and sectioned as described (Griffith et al., 2011).

Immunohistochemistry

Sections were deparafinized with three 10-min xylene washes and rehydrated with three 5-min washes in 100% ethanol, followed by successive 1-min washes in 90%, 80%, 70% ethanol and water. Antigen retrieval was performed by boiling for 5 min in 0.01 M Tris Base pH 10.0 with 0.05% Tween20. After slides cooled to room temperature they were washed twice in PBT for 2 min and blocked with 0.5% milk in PBT for 2 h at room temperature in a humidified chamber. Primary antibody was applied in 0.05% milk/PBT overnight at 4 °C in a humid chamber. Three 15-min PBT washes preceded a 1-h secondary treatment in 0.05% milk/PBT in a humid chamber at room temperature. Slides were washed in PBT for 15 min twice and then in PBS for 15 min. Nuclei were counterstained with Dapi (Roche or Molecular Probes) in PBS (1:10,000) for 2 min and then rinsed with PBS. Slides were sealed and coverslipped with Prolong Gold (Invitrogen). Primary antibodies were used at the following concentrations: YY1 [Santa Cruz, sc-1703 (1:100)], CDH1 [Abcam, ab53033 (1:500)], HNF4α [Santa Cruz sc6556 (1:200)]. Secondary antibodies were diluted 1:500 and included Alexa Fluor 488 donkey-anti-rabbit [Molecular Probes (A-21206)] and Alexa Fluor 546 donkey-anti-goat [Molecular Probes (A-11056)].

Whole-mount *in situ* hybridization

Embryos were fixed in 4%PFA/PBS overnight at 4°C with agitation, dehydrated in a series of methanol washes and stored at −20 °C. E7.5 embryos were treated with proteinase K for 10 min and embryos collected at E7.0 were treated with proteinase K for 8 min. *In situ* hybridization probe synthesis and protocols were performed as previously described (Rivera-Perez and Magnuson, 2005). WISH stained embryos were imaged in PBT. Probes used: *Brachyury* (Wilkinson et al., 1990), *Lefty2* (Meno et al., 1997), *Fgf8* (Crossley and Martin, 1995), *Bmp4* (probe produced from RT-PCR product using primers 5'AGGAGGAGGAGGAAGAGCAG3' and 5'TGTGATGAGGTG-TCCAGGA3'), *Snail*, 5'ACACTGGGTGAGAAGCCATT3' and 5'GAAGG-AGTCTGGCAGTGAG3'; *Fgf4*, 5'TTGCGTCTCTATTGCTCTC3' and 5'CGGAGGGTCACAGTCTAGGA3'; *Eomes* (Russ et al., 2000), *Nodal*

(Conlon et al., 1994), *Foxa2* (Sasaki and Hogan, 1996), *Cer1* (Thomas et al., 1997), *Hex* (Bedford et al., 1993), *Sox2* (Conlon et al., 1994), *Otx2* (gift from James Li), *Oct4* (Rosner et al., 1990), *Shh* (gift from James Li). A minimum of 3 mutants were analyzed at each stage for each *in situ* presented.

Imaging

Digital images of whole mount embryos were captured on a Nikon SMZ-1500 stereomicroscope equipped with a Spot Idea Digital Camera and Spot software (v4.6). Digital images of sectioned embryos were taken with a Nikon Eclipse TE2000-S inverted fluorescence microscope and QImaging Retiga EXi Fast 1394 camera fitted with a color-slider for use with brightfield images. All slides were imaged with NIS-Elements BR Software.

Chromatin immunoprecipitation

Putative YY1 binding sites were identified using MacVector software and searching for the YY1 consensus binding sequence as described previously by other groups (5'-(C/g/a)(G/t)(C/t/a)CATN(T/a)(T/g/c)-3' where the upper case letters represent the preferred bases) (Hyde-DeRuyscher et al., 1995; Shi et al., 1997). E7.5 embryos were collected in PBS/PVP and immediately processed with the MagnaChipA Kit (Millipore MAGNA0001) according to the manufacturer's instructions. Briefly, the embryos were treated with 1% formaldehyde at RT for 10 min, squelched with glycine and washed with cold PBS three times. E7.5 embryos were processed in groups of ten. Samples were incubated in Protease Inhibitor Cocktail with Cell Lysis Buffer on ice for 15 min, pelleted, and resuspended in 500 μ l of Protease Inhibitor Cocktail with Nuclear Lysis Buffer. Samples were kept on ice and sonicated twice for 20 s with the Heat Systems Sonicator/Ultrasonic Processor (output setting 3) generating chromatin fragments between 200–1000 bp. 1% of each

sample was removed for Input control. Immunoprecipitation was performed using 20 μ l of Magnetic Protein A Beads and 50 μ l anti-YY1 H414 antibody (Santa Cruz, sc-1703) or normal rabbit serum with overnight incubation at 4°C followed by 2 h at RT with over-end mixing. After antibody incubation samples were sequentially washed with low salt, high salt, LiCl and TE solutions, washed and collected by spin column as described in the manufacturer's directions and eluted in 50 μ l. Quantitative PCR was performed using PerfeCTa[®] qPCR SybrMix, Low ROX[™] (Quanta Biosciences) and run on a Stratagene 3001mx Q-PCR machine using Quanta's recommended cycling conditions. Primers used are shown in Sup. Fig. 3.

Whole embryo culture

E6.5 embryos were carefully dissected in pre-warmed and incubated dissection media (DMEM with 7.5% FBS) on a 37 °C warming plate under a dissecting microscope. After removal of Reichert's membrane, whole litters of embryos were cultured for 20 h in rotating tubes in 5% CO₂ 37 °C incubator in 75% rat serum/25% DMEM with or without 25 μ M SB505124 (Sigma S4696). After culture, embryos were genotyped and processed for IHC/IF or RT-PCR. At least 3 mutant embryos were examined for each data set (IHC/RT-PCR).

Results

Characterization of embryos with epiblast-specific YY1 deletion

YY1 is ubiquitously expressed in embryonic and extraembryonic tissues throughout gastrulation (Fig. 1A–C). Using a paternally inherited Sox2-Cre transgene (Hayashi et al., 2002), *Yy1* was deleted in the epiblast. Although YY1 is present throughout wild type (WT) and cKO embryos at E5.5 (Fig. 1A and D), it is absent

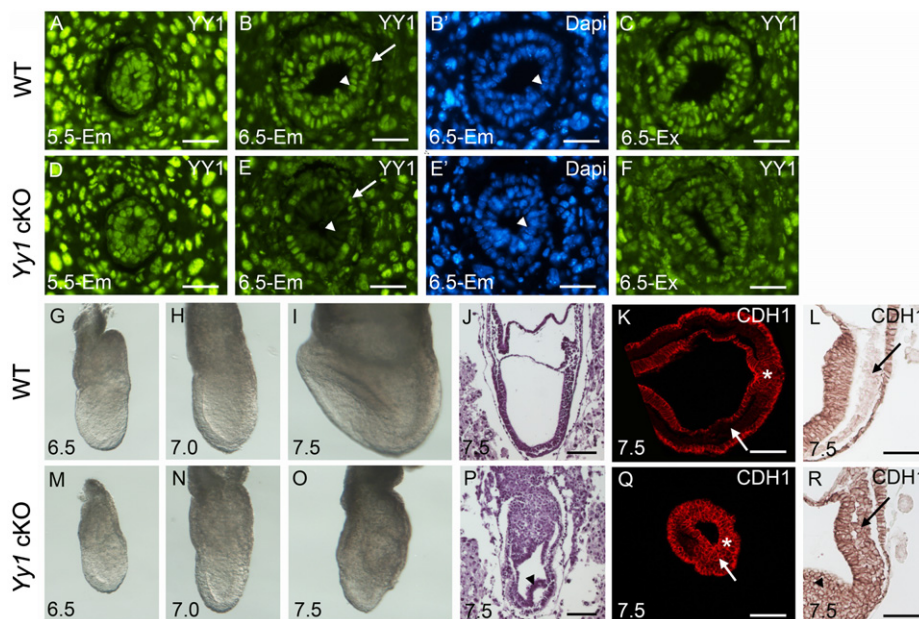


Fig. 1. YY1 is required in the epiblast for developmental EMT. YY1 (green) localization in WT (A–C) and mutant (D–F) sectioned embryos. At E5.5 (A,D), YY1 is present throughout both mutant and WT embryos. At E6.5 YY1 is expressed in the WT epiblast (arrowhead in B and B'), VE (arrow in B and B') and extraembryonic tissues (C). YY1 is absent specifically from the mutant epiblast (arrowhead in E and E') starting at E6.5. YY1 expression is unaffected in the mutant visceral endoderm (arrow in E and E') and other extraembryonic tissues (F). B' and E' are DAPI fluorescence of the same sections in B and E, respectively. Bright field images of WT (G–I) and mutant (M–O) images during gastrulation. Haematoxylin and eosin staining of sagittal E7.5 WT (J) and mutant (P) sections. Arrowhead in P and R indicates abnormal primitive streak derivatives. E-Cadherin (CDH1) localization on transverse (K and Q) and sagittal (L and R) E7.5 WT and mutant embryos as indicated. While E-Cadherin is normally present throughout the cells of the PS (asterisk, K and L), it is repressed in the streak derivatives in the WT (arrows, K and L) and remains on in the mutant PS derivatives (arrows, Q and R). Scale bars in A–F represent 25 μ m. Scale bars in J–L and P–R represent 75 μ m.

from the cKO epiblast at the onset of gastrulation at E6.5 (arrow-head in Fig. 1E and E'). As expected, YY1 expression is maintained in the cKO extraembryonic tissues (Fig. 1F), including the visceral endoderm (VE, arrow in Fig. 1E, E'). Also as predicted, YY1 is absent in the mutant epiblast and epiblast-derived tissues at E7.5 (Sup. Fig. 1).

Yy1 cKO mutant embryos are smaller than control littermates at E6.5 and E7.0 (compare Fig. 1G–M and H–N) and are easily distinguished by E7.5 due to the absence of a midline, node and head-folds as well as abnormal extraembryonic region (Fig. 1I and O). While the primitive streak (PS) is apparent in mutants, there is a distinct accumulation of cells proximal to the streak (arrow-heads, Fig. 1P and R), suggesting defects in EMT.

YY1 is required in the epiblast for EMT and PS elongation

Repression of E-Cadherin (CDH1) is an essential step of EMT, permitting movement of PS cells and their derivatives [reviewed in (Thiery et al., 2009)]. An excess of CDH1 in mutant streak derivatives (compare Fig. 1K–L with Q–R) indicates that although YY1 deficient cells are capable of delaminating from the epiblast and can ingress through the streak, they do not properly repress E-Cadherin. The retention of CDH1 in streak derivatives likely contributes to the posterior accumulation of mutant cells that fail to migrate properly and remain tightly associated.

Because of the evident morphological PS defects, we examined expression of genes known to be critical for this transient embryonic structure. In control embryos, *brachyury* (*T*) is confined to the PS and nascent mesoderm at E7.0 [Fig. 2A, (Wilkinson et al.,

1990)]. At E7.5 *T* expression continues to mark the streak as it expands distally in WT embryos (Fig. 2B), a requirement for anterior-posterior patterning and proper development (Beddington et al., 1992). At E7.0, the expression domain of *T* in Yy1 cKO embryos is reduced compared to littermate controls (compare Figs. 2A and F), and at E7.5, *T* expression has failed to extend distally (Fig. 2G). Despite the drastic reduction in overall streak size, analysis of sectioned mutant embryos reveals a population of *T* negative mesenchyme surrounding the streak, suggesting that a limited amount of cell migration through the PS has occurred (data not shown).

To further examine the PS in Yy1 cKO mutants, we examined other markers of nascent mesoderm, including *Lefty2*, *Snail*, *Fgf8* and *Fgf4* (Carver et al., 2001; Meno et al., 1999; Sun et al., 1999). *Lefty2* and *Fgf4* were completely absent in mutants at all stages examined (compare Fig. 2C–D with H–I, *Fgf4* not shown), and *Snail* and *Fgf8* expression is greatly reduced in cKO mutants, with weak areas of expression confined to the distal-most mesoderm (compare Fig. 2E and J). Because these nascent mesoderm markers are also important for EMT, loss of expression of these genes likely contributes to the EMT defects noted above [reviewed in (Arnold et al., 2008)].

Expression of *Bmp4* and *Eomes* in the extending PS is preceded by their expression in distal extraembryonic ectoderm (EXE). Concomitant with their expression in the newly formed streak, *Bmp4* and *Eomes* are normally down-regulated in EXE (Ciruna and Rossant, 1999; Fujiwara et al., 2002; Russ et al., 2000). In Yy1 mutant embryos *Bmp4* and *Eomes* show appropriate tissue specific localization. However, both genes are overexpressed compared

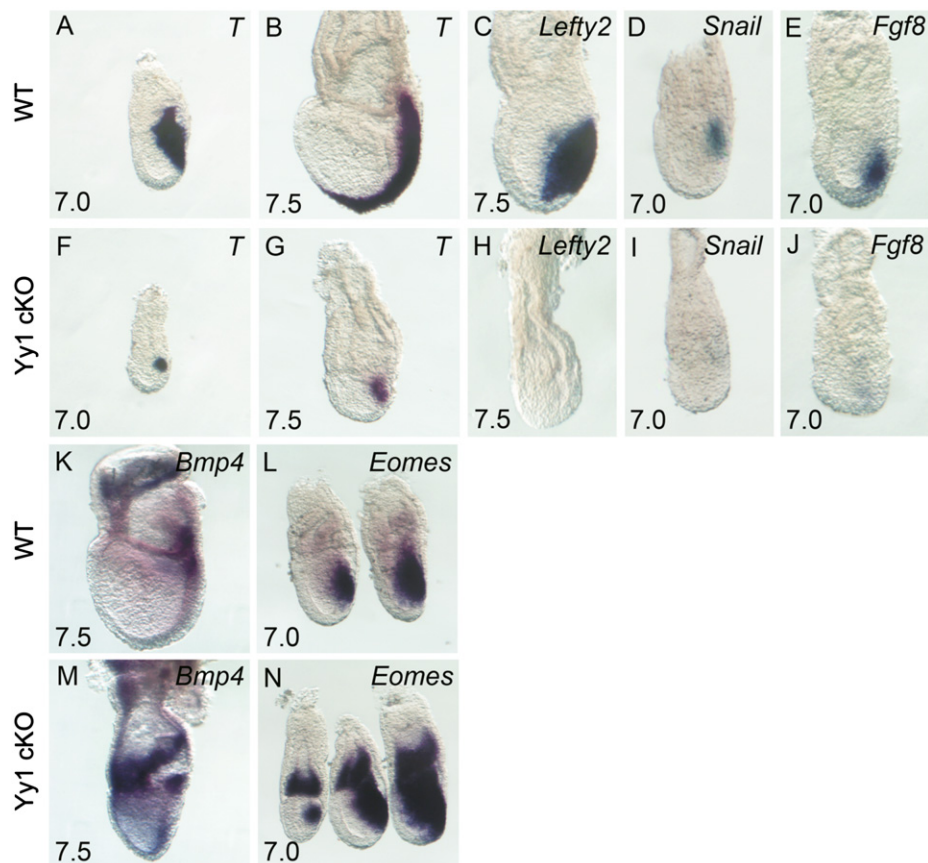


Fig. 2. YY1 is required in the epiblast for PS function and paracrine regulation. Whole mount *in situ* hybridization (WISH) of *T* (A–B, and F–G), *Lefty2* (C and H), *Snail* (D and I), *Fgf8* (E and J), *Bmp4* (K and M) and *Eomes* (L and N). Comparison of WT (A–E and K–L) and mutant embryos (F–J and M–N) reveals compromised PS formation marked by reduced *T* expression (F and G), loss of the mesoderm markers *Lefty2* (H), *Snail* (I) and *Fgf8* (J) and excess extraembryonic and embryonic *Bmp4* (M) and *Eomes* (N) in mutant embryos.

with WT embryos of the same stage (Fig. 2K–N), suggesting a failure of appropriate repression of these genes in both embryonic and extraembryonic tissues of *Yy1* cKO embryos. Disruption of gene expression in the extraembryonic region of the conceptus is unanticipated in *Yy1* mutants, as the *Yy1* locus is not deleted in EXE (Fig. 1E). However, our results are consistent with reports that signaling from embryonic ectoderm is important for proper regulation of *Bmp4* and *Eomes* in EXE [reviewed in (Arnold and Robertson, 2009)], and suggest that this paracrine epiblast activity is YY1 dependent.

Organizer formation and function in *Yy1* cKO embryos

Intact *Nodal* signaling is critical for a variety of developmental processes including the onset of gastrulation, anterior/posterior patterning, PS formation as well as both endoderm and mesoderm induction [reviewed in (Arnold et al., 2008; Lu et al., 2001; Schier, 2003)]. In accordance with multiple critical roles during development, early *Nodal* expression is dynamic and tightly regulated. *Nodal* is normally expressed throughout the epiblast prior to gastrulation and gradually becomes restricted to the PS where its expression is further confined to the anterior streak at E7.0

(Fig. 3A). By E7.5 *Nodal* is restricted to the node in WT embryos (Fig. 3B). In contrast, *Nodal* is ectopically expressed throughout the posterior half of the mutant epiblast at E7.0 (Fig. 3E), and throughout the posterior epiblast and streak derivatives by E7.5 (Fig. 3F).

High levels of *Nodal* signaling are required to specify the definitive endoderm (DE) and axial midline structures including the node and notochord (Dunn et al., 2004; Robertson et al., 2003; Tremblay et al., 2000; Vincent et al., 2003). No morphological node or notochord was evident in any mutant embryos examined. This observation is supported by the absence of *Shh* expression in cKO mutants (data not shown). In wild type E7.0 embryos, *Foxa2* expression is restricted to the anterior visceral endoderm (AVE, Fig. 3C and arrow in C') and DE (arrow in Fig. 3C and C'). In *Yy1* mutants, *Foxa2* is correctly localized to the AVE (arrow in Fig. 3G') but is aberrantly expressed throughout distally located streak derived cells (arrow in Fig. 3G''). As development progresses *Foxa2* is normally expressed in both the anterior DE and the notochord at E7.75 (Fig. 3D). In E7.5 mutants, *Foxa2* is inappropriately expressed in a large swath of distally located streak derived cells at E7.5 (Fig. 3H). Taken together these data suggest that axial midline structures are not present but that the DE is specified and abundant in *Yy1* cKO mutants.

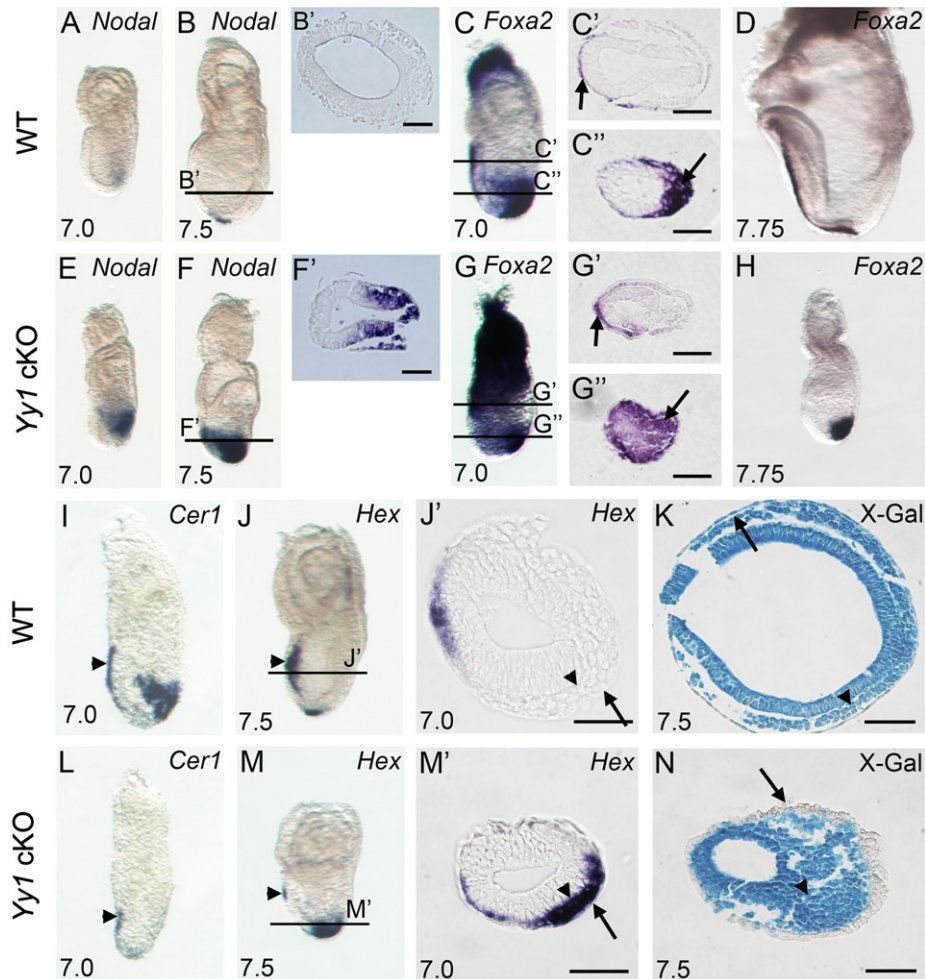


Fig. 3. Defective gastrulation in *Yy1* cKO mutants. WISH of *Nodal* (A–B and E–F) showing failure of spatiotemporal repression in *Yy1* mutants. B' and F' are transverse sections corresponding to the lines in B and F. WISH of *Foxa2* (C–D and G–H). C', C'', G' and G'' are transverse sections corresponding to the lines in C and G. WISH of *Cer1* (I and L); *Hex* (J and M) marking properly migrating AVE cells in WT (arrowhead in J) and mutant embryos (arrowhead in M). In WT embryos, *Hex* positive DE cells have intercalated into VE and have migrated anteriorly. Mutant *Hex* positive cells (arrowhead in M') fail to intercalate into VE (arrow in M'). J' and M' are transverse sections corresponding to the lines in J and M. Arrows in J' and M' indicate VE. Transverse sections of X-gal stained R26R WT (K) and mutant (N) embryos. Arrowhead in K and N indicate epiblast derivatives. Arrows in K and N indicate cells in the outer layer of the conceptus, showing β-gal positive epiblast derived DE in WT embryos and β-gal negative extraembryonic derived VE in mutants. Scale bars represent 75 μm.

Abnormal endoderm specification in *YY1* cKO Embryos

Once the streak has extended distally in WT embryos, DE gene expression can be observed [reviewed in (Lewis and Tam, 2006)] as streak derived DE cells first intercalate into the visceral endoderm (Kwon et al., 2008). To assess DE specification, we examined markers including *Cer1*, *Hex* and *Foxa2*. In *Yy1* mutants, *Cer1* is expressed in the AVE (arrowhead Fig. 3L), but is completely absent in streak derivatives (compare Figs. 3I and L). *Hex* is expressed in the mutant AVE (arrowhead Fig. 3M), but unlike *Cer1*, *Hex* is also expressed in streak derivatives that remain in the middle germ layer instead of migrating/intercalating into the outermost layer (compare Figs. 3J' and M'). Combined with the *Foxa2* *in situ* results discussed above (Fig. 3G'' and H), this data suggests that loss of *YY1* in the epiblast leads to endoderm specification without the ability to intercalate into the outer layer.

To examine the lack of DE intercalation, we first utilized the R26R allele to lineage trace Sox2-Cre positive epiblast cells. As expected, all three germ layers of WT *Sox2-Cre*^{+/−}; R26R^{+/−} E7.5 embryos showed X-gal activity indicating appropriate movement of epiblast derived DE into the outer layer (arrow in Fig. 3K). In contrast, X-gal positive cells were not present in the outer layer of mutant embryos, supporting the hypothesis that epiblast-derived cells do not properly integrate into the outer layer in mutant embryos (arrow in Fig. 3N). To assess the identity of cells surrounding the mutant epiblast, HNF4α expression was examined. In WT embryos, HNF4α positive visceral endoderm (VE) surrounds only the extraembryonic tissues of the conceptus [Sup. Fig. 2A–E and (Duncan et al., 1994)]. However, in mutant embryos, the thickened HNF4α positive VE completely surrounds the conceptus (Sup. Fig. 2F–J) indicating an absence of the DE-mediated dispersal of VE. Taken together, these data show that in the absence of *YY1*, the DE is specified but does not intercalate into the surrounding HNF4α expressing VE.

Anterior/posterior patterning in *Yy1* mutant embryos

The AVE is a subset of VE initially located at the distal tip of the egg cylinder at E5.5 that migrates anteriorly prior to the onset of gastrulation (Beddington and Robertson, 1999; Rivera-Perez et al., 2003). Anterior specification is dependent on both AVE migration and the subsequent maintenance of AVE specific gene expression, events coordinated by reciprocal interactions between the AVE and epiblast [reviewed in (Lu et al., 2001)]. As discussed above, AVE expression of both *Cer1* and *Hex* is appropriately localized in E7.0 *Yy1* cKO mutants,

indicating that the AVE has migrated anteriorly. However, expression of both genes is significantly reduced, again implicating *YY1* in embryonic (epiblast) to extraembryonic (AVE) signaling (compare Fig. 3I–J with L–M).

From E7.0–7.5 the expression domains of *Sox2*, *Otx4* and *Otx2* normally overlap in the WT prospective anterior ectoderm, specifying presumptive neural progenitors [Fig. 4A–E (Scholer et al., 1990; Wood and Episkopou, 1999)]. Surprisingly, at E7.0 and E7.5 appropriate *Sox2* and *Otx4* expression is observed in mutant embryos (Fig. 4). However, the *Otx2* expression domain is reduced at E7.0 and fails to become anteriorly restricted (compare Fig. 4C and H). At E7.5 *Otx2* is confined to a small population of distal epiblast cells in mutant embryos (compare Fig. 4D and I), results consistent with data demonstrating that *YY1* activates the *Otx2* locus (Takasaki et al., 2007). However, the normal *Otx4* and *Sox2* expression indicate that neural specification is initiated in the absence of embryonic *YY1*.

YY1 binds directly to the *Lefty2* locus *in vivo*

As many of the phenotypic features of the *Yy1* cKO embryo could be attributed to increased Nodal signaling, we next chose to examine if *YY1* interacted with either the *Nodal* or *Lefty2* locus in E7.5 *in vivo* by chromatin immunoprecipitation (ChIP). Several predicted *YY1* binding sites (Fig. 5A and S3) at the *Lefty2* locus were examined and specific binding of *YY1* to sequences within intron 3 (Fig. 5B) was found in E7.5 chromatin. Importantly, we detected no/weak interaction at intron 2 of *Lefty2* (F2R2, Fig. 5A–B), indicating specificity of *YY1* binding. Despite a similar experimental approach, specific interactions at the *Nodal* locus were not detected in E7.5 chromatin (data not shown). Taken together, our *in situ* and ChIP results suggest that in the gastrulating epiblast *YY1* may bind to and activate *Lefty2* transcription, which in turn represses *Nodal* [Fig. 5C, (Meno et al., 1999)].

Nodal reduction rescues mesoderm induction in *Yy1* cKO embryos

Regulation of Nodal signaling is critical for many aspects of early embryonic development (Brennan et al., 2001; Conlon et al., 1994; Meno et al., 1997). To assess the role of *Nodal* overexpression in *Yy1* mutant embryos, we suppressed Nodal activity by pharmacological inhibition with SB505124, an inhibitor of TGFβ type 1 receptor signaling (DaCosta Byfield et al., 2004). We found that 25 μM SB505124 was sufficient to block Nodal signaling in WT embryos as evidenced by a reduction in *Nodal* transcripts [due to the absence of

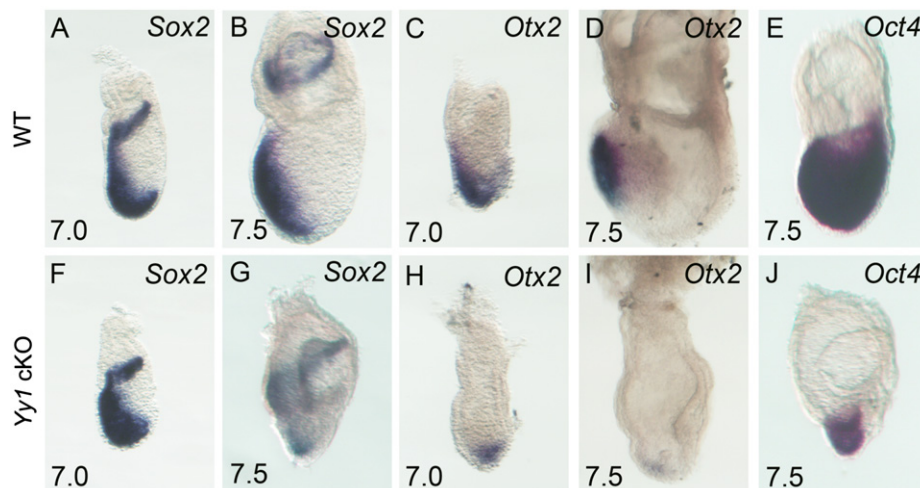


Fig. 4. Neural progenitor specification is initiated in *Yy1* cKO embryos. WISH of *Sox2* (A, B, F, G), *Otx2* (C, D, H, I) and *Otx4* (E, J) in WT (A–E) and mutant embryos (F–J) at E7.0 (A, C, F, H) and E7.5 (B, D, E, G, I, J). Anterior *Sox2* and *Otx4* expressing cells demonstrate specification of neural progenitors, despite the drastic reduction in *Otx2* expression.

Nodal autoregulation (Brennan et al., 2001; Norris et al., 2002)], but still allows for embryo survival in culture (Fig. S4). E6.5 litters were dissected and cultured for 24 h with or without SB505124. *Yy1* cKO

embryos cultured with SB505124 showed an obvious increase in *YY1* negative (epiblast derived) mesenchymal cells surrounding the epiblast (Fig. 5D, asterisks) suggesting an increase in the movement of cells through the streak. To further evaluate the identity of these cells, we assessed expression of transcripts that are altered in the absence of *YY1*.

Further indicating enhanced mesoderm specification, analysis of SB505124 treated cKO embryos demonstrated a slight increase in *T* expression as well as a dramatic increase in *Tbx6*, *Twist1* and *Snail* transcripts (Fig. 5E–F), which are all severely reduced or absent in untreated mutant embryos. Consistent with findings that *Foxa2* and *Eomes* expression levels correlate with *Nodal* activity (Arnold et al., 2008; Vincent et al., 2003), treatment of *Yy1* cKO embryos with SB505124 resulted in reduced expression of both genes (Fig. 5E–F). Importantly, reduction of *Nodal* signaling had no effect on the loss of *Lefty2* in cKO embryos, supporting a direct role for *YY1* in the regulation of *Lefty2* that is independent of *Nodal* activity. Likewise, SB505124 treatment of cKO embryos had no effect on the loss of *Fgf4* in cKO embryos, indicating that both *Nodal* dependant and *Nodal* independent events contribute to the gastrulation defects occurring in the absence of *YY1* in the epiblast.

Discussion

Here we present the results of an epiblast-specific deletion of *Yy1*, a multifunctional gene that has a plethora of functions ascribed to it in many cell types *in vitro*. *YY1* is expressed throughout the epiblast and extraembryonic regions of the embryo prior to and during gastrulation. *Sox2*-Cre mediated deletion of *Yy1* leads to a loss of *YY1* protein throughout the epiblast. Mutant embryos display noticeable phenotypes by E7.0, indicating that *YY1* is critical for gastrulation. Two main observations can be made from the analysis of E7.0–7.5 mutant embryos. The first is that although PS formation is initiated, it does not extend anteriorly and the streak derivatives fail to repress E-Cadherin resulting in an accumulation of streak derivatives. Secondly, *Nodal* fails to undergo proper spatiotemporal regulation and remains expressed throughout much of the epiblast at E7.5. We suggest that while many aspects of the cKO phenotype can be attributed to over expression of *Nodal* (due to the absence of *Lefty2* activation by *YY1*), other *Nodal*-independent transcriptional and morphogenetic abnormalities (loss of *Fgf4* and PS defects) also occur when *YY1* is not present in the epiblast.

Critical requirement of *YY1* in EMT

The most obvious morphological feature of *Yy1* cKO embryos is the accumulation of PS cells that fail to migrate properly. The retention of E-Cadherin by these streak derivatives causes reduced mobility which in turn presumably abrogates (1) the migration of

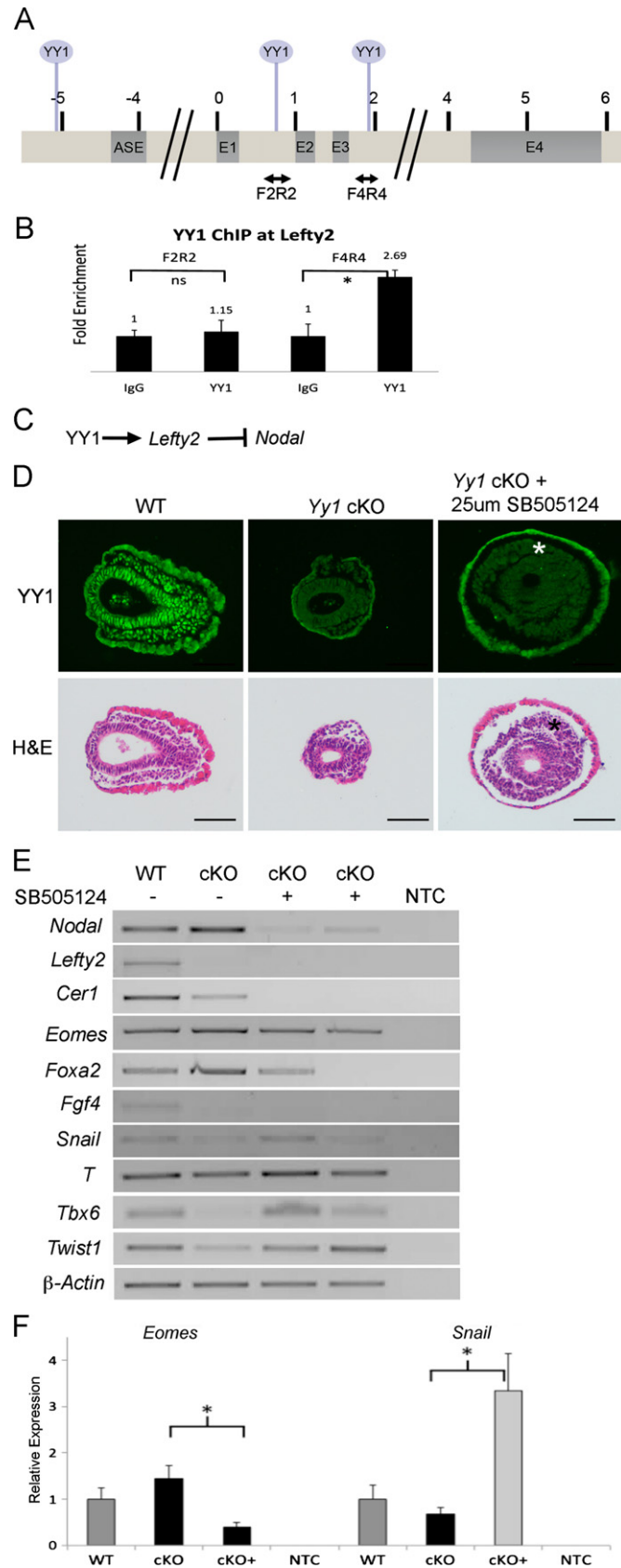


Fig. 5. *YY1* regulates *Nodal* indirectly through *Lefty2*. (A) Map of the *Lefty2* locus. Double-headed arrows indicate amplicons used for *YY1* ChIP and circles above mark locations of predicted *YY1* binding sites. (B) *YY1* ChIP-qPCR shows that *YY1* binds between exons 3 and 4 (F4R4) *in vivo*, but not between exons 1 and 2. (C) Schematic illustration of possible regulation of *Nodal* through activation of *Lefty2*. (D) Transverse sections of untreated wildtype and mutant embryos, as well as mutant embryos cultured with the TGFβ inhibitor SB505124 (top row is *YY1* immunofluorescence and bottom row is H&E of the same sections). Treatment with SB505124 results in increased mesodermal-like streak derivatives in mutant embryos (asterisks). (E) RT-PCR indicates that mesodermal markers *Tbx6*, *Twist1* and *Snail* are increased in the mutants treated with the inhibitor while *Lefty2* and *Fgf4* expression are unaffected as predicted. RT-PCR also confirms reduction of *Nodal*, *Cer1*, *Eomes* and *Foxa2* by treatment with SB505124. (F) RT-qPCR confirmation of reduced *Eomes* and enhanced *Snail* expression in mutant embryos treated with SB505124. Asterisks represent *P* values less than 0.05 by student's *T*-Test. Error bars represent SEM. Scale bars represent 75 μ m.

mesoderm; (2) the proximal displacement of EXE; and (3) DE intercalation into the overlying VE.

Functionally redundant pathways are known to regulate EMT, and while some mechanisms are cell type specific, most utilize a conserved pathway involving repression of E-Cadherin by a member of the Slug family (Barrallo-Gimeno and Nieto, 2005). Many cofactors have been shown to be required for Slug's regulation of E-Cadherin. These include AJUBA, SUZ12 and the SIN3A/HDAC1/HDAC2 histone-modifying complex (Calder et al., 2008; Herranz et al., 2008; Peinado et al., 2004). In mouse the *Slug* homolog *Snail* represses E-Cadherin in cells undergoing EMT during gastrulation, allowing PS formation to proceed (Sefton et al., 1998). Similar to what is found in *Yy1* cKO embryos, *Snail* null mouse embryos do not complete gastrulation and show aberrant E-Cadherin expression in streak derivatives (Carver et al., 2001), suggesting that one role of *YY1* is to activate *Snail* in the PS. Indeed, *YY1* has been shown to bind the *Snail* enhancer and activate the locus in HeLa cells (Palmer et al., 2009), supporting the hypothesis that *YY1* may be an activator of the *Snail* locus *in vivo* as well. Furthermore similar to several mouse mutants in which EMT is disrupted, the PS forms in *Yy1* cKO embryos but fails to extend appropriately (Arnold et al., 2008; Carver et al., 2001; Ciruna and Rossant, 2001; Sun et al., 1999). Additionally, both *Fgf4* and *Fgf8* are known to be required for *Snail* expression (Ciruna and Rossant, 2001), and transcripts from both of these genes are absent in *Yy1* mutants. These divergent mechanisms regulating *Snail* and EMT are not mutually exclusive, making it difficult to assess if loss of *Snail* is a direct or indirect consequence of cells lacking *YY1*.

YY1 mediates Nodal signaling

One exciting molecular phenotype apparent in *Yy1* cKO mutant embryos is the mis-regulation of *Nodal* signaling. Appropriate spatiotemporal *Nodal* expression is critical for many aspects of normal development. Deletion of *Yy1* leads to an expansion of *Nodal* throughout the epiblast at E7.5 with concomitant loss of two antagonists of the nodal pathway, *Cer1* and *Lefty2*. It is important to note that loss of *Lefty2* alone results in accumulation of PS cells, lack of axial midline structures and increased *Foxa2* expression (Meno et al., 1999) – features that we also document in *Yy1* cKO mutants (Figs. 1–3). Furthermore, combined loss of *Cer1* and *Lefty1*, has been shown to result in an accumulation of cells in the streak and an increase in endodermal gene expression (Perea-Gomez et al., 2002; Yamamoto et al., 2004). The similarities of gastrulation phenotypes between *Yy1* cKO and *Nodal* antagonist mutant embryos suggest that mis-regulation of *Nodal* may be one of the primary molecular defects underlying the gastrulation phenotype we observe. Consistent with this possibility, our ChIP results indicate that *YY1* binds directly to the *Lefty2* locus in E7.5 embryos (Fig. 5A–B), suggesting that *YY1* normally activates *Lefty2* and indirectly regulates *Nodal* signals (Fig. 5C). Additionally, we find that the suppression of *Nodal* in the context of a *YY1* null epiblast results in enhanced mesoderm formation and increased expression of mesodermal markers including *Tbx6*, *Twist* and *Snail* (Fig. 5E and F). Furthermore, we find that the expression of *Foxa2* and *Eomes* in cKO embryos correlate with *Nodal* levels, suggesting that overexpression of these genes in the cKO embryo is a result of *Nodal* overexpression. These results support the hypothesis that over-expression of *Nodal* signaling is an important component of the cKO phenotype and suggest that one role of *YY1* may be repression of *Nodal* through activation of *Lefty2*.

Several reports have shown that differential post-translational modifications of *YY1* can alter binding specificity (Hiromura et al., 2003; Takasaki et al., 2007; Yao et al., 2001; Zheng et al., 2010). Therefore we cannot rule out the possibility that *YY1* may also act as a direct repressor of *Nodal*. It may be informative to determine precisely which post-translationally modified *YY1* proteins are

present in the epiblast, distinct germ layers and organizing centers during development and differentiation.

Despite the finding that anterior DE markers are specified, we observe a lack of intercalation of *YY1* negative cells into the VE layer in cKO embryos (Fig. 3 and S2). A similar phenotype is found in other mutations that down-regulate FGF signaling in the streak (Ciruna et al., 1997; Sun et al., 1999), suggesting that in *YY1* mutants this phenotype may be due to the observed loss of *FGF4/8* (Sun et al., 1999). The similarities between *Fgf8* knockout embryos and *Yy1* epiblast mutants suggest that loss of *Fgf8* or *Fgf4* may also be a major cause of the observed *Yy1* cKO phenotype (in addition to elevated *Nodal* signaling). *In silico* analyses indicate an absence of *YY1* binding sites at the *Fgf8* locus (data not shown). Therefore we do not believe that *YY1* is directly responsible for the loss of *Fgf8* expression, but that there may be a molecular intermediate between *YY1* and regulation of FGF signaling.

Reduction of *Nodal* in the cKO embryos had a noticeable effect on many genes implicated in primitive streak formation and mesoderm induction but did not change the level of *Fgf4*, which is absent in all cKO embryos examined. This result raises the interesting possibility that *Fgf4* is directly downstream of *YY1*. Loss of *Fgf4* results in a perimplantation phenotype that is similar to the *YY1* null embryos (Donohoe et al., 1999; Tanaka et al., 1998), suggesting that loss of *Fgf4* may in part explain the *YY1* null phenotype as well.

Eomes is normally expressed in the EXE prior to gastrulation and then in the PS as it elongates (Ciruna and Rossant, 1999; Russ et al., 2000). Studies in the mouse have demonstrated that *Nodal* signaling from the epiblast is required to maintain *Eomes* in the EXE (Brennan et al., 2001; Guzman-Ayala et al., 2004). The data we present supports this hypothesis, demonstrating that loss of *YY1* in the epiblast leads to prolonged high levels of epiblast derived *Nodal* signaling, which subsequently produces high levels of *Eomes* in the adjacent EXE. *Eomes*, like *Nodal*, is required for DE specification in the epiblast (Arnold and Robertson, 2009) and although both factors are required for proper gastrulation, the extent to which they interact in the proximal epiblast and PS is unclear. Our study supports a role for *YY1* in the negative regulation of *Nodal* and supports the idea that alterations in *Eomes* expression are downstream of changes in *Nodal* expression (Figs. 2, 3 and 5).

Also of note are findings that DRAP1, a transcriptional co-repressor, prevents excess *Nodal* accumulation in the epiblast (Iratni et al., 2002). Through interactions with FOXH1, DRAP1 attenuates the positive *Nodal* auto-regulatory feedback loop, such that loss of *Drap1* results in increased expression of *Nodal*. Similar to *Yy1* cKO embryos, *Drap1* mutants demonstrate an accumulation of cells adjacent to the streak, an abundance of *Nodal* in the epiblast, dramatic reduction of *T*, an increase in *FoxA2* and a loss of *Lefty2*. Furthermore, reduction of *Nodal* in *Drap1* null embryos restores *T* expression, similar to what we present with *YY1* cKO mutants (Fig. 5). *Drap1* is expressed specifically in the epiblast during gastrulation. We observe no alteration in *Drap1* mRNA levels in *Yy1* cKO embryos (data not shown) raising the possibility that *YY1* may physically interact with DRAP1 in the epiblast.

In conclusion we show that *YY1* is required *in vivo* for proper morphogenetic movements during gastrulation as well as to maintain appropriate spatiotemporal *Nodal* expression, possibly through activation of *Lefty2*.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2012.05.031>.

References

- Affar el, B., Gay, F., Shi, Y., Liu, H., Huarte, M., Wu, S., Collins, T., Li, E., 2006. Essential dosage-dependent functions of the transcription factor yin yang 1 in late embryonic development and cell cycle progression. *Mol. Cell Biol.* 26, 3565–3581.
- Arnold, S.J., Hofmann, U.K., Bikoff, E.K., Robertson, E.J., 2008. Pivotal roles for eomesodermin during axis formation, epithelium-to-mesenchyme transition and endoderm specification in the mouse. *Development* 135, 501–511.
- Arnold, S.J., Robertson, E.J., 2009. Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat. Rev. Mol. Cell Biol.* 10, 91–103.
- Atchison, L., Ghias, A., Wilkinson, F., Bonini, N., Atchison, M.L., 2003. Transcription factor YY1 functions as a PcG protein *in vivo*. *Embo. J.* 22, 1347–1358.
- Barrallo-Gimeno, A., Nieto, M.A., 2005. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132, 3151–3161.
- Beddington, R.S., Rashbass, P., Wilson, V., 1992. Brachyury—a gene affecting mouse gastrulation and early organogenesis. *Dev. Suppl.*, 157–165.
- Beddington, R.S., Robertson, E.J., 1999. Axis development and early asymmetry in mammals. *Cell* 96, 195–209.
- Bedford, F.K., Ashworth, A., Enver, T., Wiedemann, L.M., 1993. HEX: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res.* 21, 1245–1249.
- Brennan, J., Lu, C.C., Norris, D.P., Rodriguez, T.A., Beddington, R.S., Robertson, E.J., 2001. Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* 411, 965–969.
- Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L., Kassiss, J.A., 1998. The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. *Mol. Cell* 1, 1057–1064.
- Calder, M.D., Madan, P., Watson, A.J., 2008. Bovine oocytes and early embryos express Staufin and ELAVL RNA-binding proteins. *Zygote* 16, 161–168.
- Carver, E.A., Jiang, R., Lan, Y., Oram, K.F., Gridley, T., 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol. Cell Biol.* 21, 8184–8188.
- Ciruna, B., Rossant, J., 2001. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* 1, 37–49.
- Ciruna, B.G., Rossant, J., 1999. Expression of the T-box gene Eomesodermin during early mouse development. *Mech. Dev.* 81, 199–203.
- Ciruna, B.G., Schwartz, L., Harpal, K., Yamaguchi, T.P., Rossant, J., 1997. Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* 124, 2829–2841.
- Conlon, F.L., Lyons, K.M., Takaes, N., Barth, K.S., Kispert, A., Hermann, B., Robertson, E.J., 1994. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* 120, 1919–1928.
- Crossley, P.H., Martin, G.R., 1995. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* 121, 439–451.
- DaCosta Byfield, S., Major, C., Laping, N.J., Roberts, A.B., 2004. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. *Mol. Pharmacol.* 65, 744–752.
- Dong, X.P., Pfister, H., 1999. Overlapping YY1- and aberrant SP1-binding sites proximal to the early promoter of human papillomavirus type 16. *J. Gen. Virol.* 80, 2097–2101, Pt 8.
- Donohoe, M.E., Zhang, X., McGinnis, L., Biggers, J., Li, E., Shi, Y., 1999. Targeted disruption of mouse Yin Yang 1 transcription factor results in peri-implantation lethality. *Mol. Cell Biol.* 19, 7237–7244.
- Duncan, S.A., Manova, K., Chen, W.S., Hoodless, P., Weinstein, D.C., Bachvarova, R.F., Darnell Jr., J.E., 1994. Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. *Proc. Natl. Acad. Sci. USA* 91, 7598–7602.
- Dunn, N.R., Vincent, S.D., Oxburgh, L., Robertson, E.J., Bikoff, E.K., 2004. Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. *Development* 131, 1717–1728.
- Fritsch, C., Brown, J.L., Kassiss, J.A., Muller, J., 1999. The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a Drosophila homeotic gene. *Development* 126, 3905–3913.
- Fujiwara, T., Dehart, D.B., Sulik, K.K., Hogan, B.L., 2002. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. *Development* 129, 4685–4696.
- Galvin, K.M., Shi, Y., 1997. Multiple mechanisms of transcriptional repression by YY1. *Mol. Cell Biol.* 17, 3723–3732.
- Garcia, E., Marcos-Gutierrez, C., del Mar Lorente, M., Moreno, J.C., Vidal, M., 1999. RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. *Embo. J.* 18, 3404–3418.
- Girton, J.R., Jeon, S.H., 1994. Novel embryonic and adult homeotic phenotypes are produced by pleiohomeotic mutations in Drosophila. *Dev. Biol.* 161, 393–407.
- Griffith, G.J., Trask, M.C., Hiller, J., Walentuk, M., Pawlak, J.B., Tremblay, K.D., Mager, J., 2011. Yin-yang1 is required in the Mammalian oocyte for follicle expansion. *Biol. Reprod.* 84, 654–663.
- Guzman-Ayala, M., Ben-Haim, N., Beck, S., Constam, D.B., 2004. Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. *Proc. Natl. Acad. Sci. USA* 101, 15656–15660.
- Hayashi, S., Lewis, P., Pevny, L., McMahon, A.P., 2002. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. *Mech. Dev.* 119 (Suppl. 1), S97–S101.
- He, Y., Casaccia-Bonnel, P., 2008. The Yin and Yang of YY1 in the nervous system. *J. Neurochem.* 106, 1493–1502.
- He, Y., Kim, J.Y., Dupree, J., Tewari, A., Melendez-Vasquez, C., Svaren, J., Casaccia, P., 2010. Yy1 as a molecular link between neuregulin and transcriptional modulation of peripheral myelination. *Nat. Neurosci.* 13, 1472–1480.
- Herranz, N., Pasini, D., Diaz, V.M., Franci, C., Gutierrez, A., Dave, N., Escriva, M., Hernandez-Munoz, I., Di Croce, L., Helin, K., Garcia de Herreros, A., Peiro, S., 2008. Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol. Cell Biol.* 28, 4772–4781.
- Hiromura, M., Choi, C.H., Sabourin, N.A., Jones, H., Bachvarov, D., Usheva, A., 2003. YY1 is regulated by O-linked N-acetylglucosaminylation (O-glcNAcylation). *J. Biol. Chem.* 278, 14046–14052.
- Hyde-DeRuyscher, R.P., Jennings, E., Shenk, T., 1995. DNA binding sites for the transcriptional activator/repressor YY1. *Nucleic Acids Res.* 23, 4457–4465.
- Iratni, R., Yan, Y.T., Chen, C., Ding, J., Zhang, Y., Price, S.M., Reinberg, D., Shen, M.M., 2002. Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. *Science* 298, 1996–1999.
- Kwon, G.S., Viotti, M., Hadjantonakis, A.K., 2008. The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extra-embryonic lineages. *Dev. Cell* 15, 509–520.
- Lee, J.S., Galvin, K.M., Shi, Y., 1993. Evidence for physical interaction between the zinc-finger transcription factors YY1 and Sp1. *Proc. Natl. Acad. Sci. USA* 90, 6145–6149.
- Lewis, S.L., Tam, P.P., 2006. Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. *Dev. Dyn.* 235, 2315–2329.
- Liu, H., Schmidt-Supplian, M., Shi, Y., Hobeika, E., Barteneva, N., Jumaa, H., Pelanda, R., Reth, M., Skok, J., Rajewsky, K., 2007. Yin Yang 1 is a critical regulator of B-cell development. *Genes Dev.* 21, 1179–1189.
- Lu, C.C., Brennan, J., Robertson, E.J., 2001. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr. Opin. Genet. Dev.* 11, 384–392.
- Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W.S., Robertson, E.J., Schier, A.F., Hamada, H., 1999. Mouse Lefty2 and zebrafish antiin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol. Cell* 4, 287–298.
- Meno, C., Ito, Y., Saijoh, Y., Matsuda, Y., Tashiro, K., Kuhara, S., Hamada, H., 1997. Two closely-related left-right asymmetrically expressed genes, lefty-1 and lefty-2: their distinct expression domains, chromosomal linkage and direct neuralizing activity in Xenopus embryos. *Genes Cells* 2, 513–524.
- Mohd-Sarip, A., Venturini, F., Chalkley, G.E., Verrijzer, C.P., 2002. Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. *Mol. Cell Biol.* 22, 7473–7483.
- Morgan, M.J., Woltering, J.M., In der Rieden, P.M., Durston, A.J., Thiery, J.P., 2004. YY1 regulates the neural crest-associated slug gene in Xenopus laevis. *J. Biol. Chem.* 279, 46826–46834.
- Norris, D.P., Brennan, J., Bikoff, E.K., Robertson, E.J., 2002. The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo. *Development* 129, 3455–3468.
- Palmer, M.B., Majumder, P., Cooper, J.C., Yoon, H., Wade, P.A., Boss, J.M., 2009. Yin yang 1 regulates the expression of snail through a distal enhancer. *Mol. Cancer Res.* 7, 221–229.
- Peinado, H., Ballestar, E., Esteller, M., Cano, A., 2004. Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol. Cell Biol.* 24, 306–319.
- Perea-Gomez, A., Vella, F.D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H., Behringer, R.R., Ang, S.L., 2002. Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev. Cell* 3, 745–756.
- Rezaei-Zadeh, N., Zhang, X., Namour, F., Fejer, G., Wen, Y.D., Yao, Y.L., Gyory, I., Wright, K., Seto, E., 2003. Targeted recruitment of a histone H4-specific methyltransferase by the transcription factor YY1. *Genes Dev.* 17, 1019–1029.
- Rivera-Perez, J.A., Mager, J., Magnuson, T., 2003. Dynamic morphogenetic events characterize the mouse visceral endoderm. *Dev. Biol.* 261, 470–487.
- Rivera-Perez, J.A., Magnuson, T., 2005. Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Dev. Biol.* 288, 363–371.
- Robertson, E.J., Norris, D.P., Brennan, J., Bikoff, E.K., 2003. Control of early anterior-posterior patterning in the mouse embryo by TGF-beta signalling. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 1351–1357, discussion 1357.
- Rosner, M.H., Viganò, M.A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W., Staudt, L.M., 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* 345, 686–692.
- Russ, A.P., Wattler, S., Colledge, W.H., Aparicio, S.A., Carlton, M.B., Pearce, J.J., Barton, S.C., Surani, M.A., Ryan, K., Nehls, M.C., Wilson, V., Evans, M.J., 2000. Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature* 404, 95–99.

- Sasaki, H., Hogan, B.L., 1996. Enhancer analysis of the mouse HNF-3 beta gene: regulatory elements for node/notochord and floor plate are independent and consist of multiple sub-elements. *Genes Cells* 1, 59–72.
- Schier, A.F., 2003. Nodal signaling in vertebrate development. *Annu. Rev. Cell Dev. Biol.* 19, 589–621.
- Schlisio, S., Halperin, T., Vidal, M., Nevins, J.R., 2002. Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. *Embo. J.* 21, 5775–5786.
- Scholer, H.R., Dressler, G.R., Balling, R., Rohdewohld, H., Gruss, P., 1990. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO. J.* 9, 2185–2195.
- Sefton, M., Sanchez, S., Nieto, M.A., 1998. Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo. *Development* 125, 3111–3121.
- Shi, Y., Lee, J.S., Galvin, K.M., 1997. Everything you have ever wanted to know about Yin Yang 1. *Biochim. Biophys. Acta* 1332, F49–66.
- Shi, Y., Seto, E., Chang, L.S., Shenk, T., 1991. Transcriptional repression by YY1, a human GLI-Kruppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* 67, 377–388.
- Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* 21, 70–71.
- Sun, X., Meyers, E.N., Lewandoski, M., Martin, G.R., 1999. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev.* 13, 1834–1846.
- Takasaki, N., Kurokawa, D., Nakayama, R., Nakayama, J., Aizawa, S., 2007. Acetylated YY1 regulates Otx2 expression in anterior neuroectoderm at two cis-sites 90 kb apart. *EMBO J.* 26, 1649–1659.
- Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., Rossant, J., 1998. Promotion of trophoblast stem cell proliferation by FGF4. *Science* 282, 2072–2075.
- Thiery, J.P., Acloque, H., Huang, R.Y., Nieto, M.A., 2009. Epithelial-mesenchymal transitions in development and disease. *Cell* 139, 871–890.
- Thomas, P., Brickman, J.M., Popperl, H., Krumlauf, R., Beddington, R.S., 1997. Axis duplication and anterior identity in the mouse embryo. *Cold Spring Harb. Symp. Quant. Biol.* 62, 115–125.
- Tremblay, K.D., Hoodless, P.A., Bikoff, E.K., Robertson, E.J., 2000. Formation of the definitive endoderm in mouse is a Smad2-dependent process. *Development* 127, 3079–3090.
- Vincent, S.D., Dunn, N.R., Hayashi, S., Norris, D.P., Robertson, E.J., 2003. Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev.* 17, 1646–1662.
- Wilkinson, D.G., Bhatt, S., Herrmann, B.G., 1990. Expression pattern of the mouse T gene and its role in mesoderm formation. *Nature* 343, 657–659.
- Wood, H.B., Episkopou, V., 1999. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech. Dev.* 86, 197–201.
- Wu, S., Hu, Y.C., Liu, H., Shi, Y., 2009. Loss of YY1 impacts the heterochromatic state and meiotic double-strand breaks during mouse spermatogenesis. *Mol. Cell Biol.* 29, 6245–6256.
- Yakovleva, T., Kolesnikova, L., Vukojevic, V., Gileva, I., Tan-No, K., Austen, M., Luscher, B., Ekstrom, T.J., Terenius, L., Bakalkin, G., 2004. YY1 binding to a subset of p53 DNA-target sites regulates p53-dependent transcription. *Biochem. Biophys. Res. Commun.* 318, 615–624.
- Yamamoto, M., Saijoh, Y., Perea-Gomez, A., Shawlot, W., Behringer, R.R., Ang, S.L., Hamada, H., Meno, C., 2004. Nodal antagonists regulate formation of the anteroposterior axis of the mouse embryo. *Nature* 428, 387–392.
- Yao, Y.L., Yang, W.M., Seto, E., 2001. Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell Biol.* 21, 5979–5991.
- Zheng, H., Chu, J., Zeng, Y., Loh, H.H., Law, P.Y., 2010. Yin Yang 1 phosphorylation contributes to the differential effects of mu-opioid receptor agonists on microRNA-190 expression. *J. Biol. Chem.* 285, 21994–22002.